THE CELL ENVELOPE AS A BARRIER TO GENETIC EXCHANGE

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Abbreviations

µL	microlitre
mL	millilitre
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetate
°C	degrees Celsius
Hfr	high frequency of recombination
LBH	Luria Bertani Herschfield broth
TNB	tris-HCl buffer
OD	optical density
g	acceleration due to gravity

Abstract

A change in the restriction phenotype of *Salmonella typhimurium* was associated with mating at 43°C. Elevated mating temperatures relaxed the barriers to gene transmission from *Escherichia coli*, increasing the frequency of IncF and IncP plasmid transmission by as much as 10^3-fold. An interesting feature of these results was that the effect on *S. typhimurium* was transient; only when mating took place at 43°C did plasmid transmission frequency increase. The rapid reversion following return to 37°C contrasts with previously reported results which suggested that the change in *S. typhimurium* restriction was prolonged, lasting up to 50 minutes after exposure to 50 °C. Furthermore, the increase in plasmid transmission at 43 °C, and the subsequent return of the restriction barrier at 37 °C, were both shown to occur independently of protein synthesis. The elevated mating temperature also reversed the poor recipient ability of an *rfaC* mutant, arguing against the involvement of a cytoplasmic nuclease. In addition, treating recipients with $CaCl_2$ prior to mating enhanced the transmission of genetic material. The combination of these results suggests involvement of the cell envelope in the increase in plasmid transmission at 43°C. The affect of ampicillin on the rate of cell fusion, an experimental system designed to test whether antibiotics can potentiate genetic exchange by removing cell surface structures that inhibit fusion, was not completed due to time constraints. Efficient conversion of cells to spheroplasts by ampicillin was, nevertheless, observed. The relevance of these findings with regard to the dissemination of antibiotic resistance is discussed.

CHAPTER I

INTRODUCTION

1.1 GENETIC EXCHANGE OF ANTIBIOTIC RESISTANCE DETERMINANTS

Antibiotic resistance gene dissemination is of intense concern to the scientific, medical and general communities (Koshland, 1994, Neu, 1992, Goldmann et al, 1996). Pathogenic bacteria are becoming resistant to antibiotics at an alarming rate (Goldmann et al, 1996). Of major relevance to this observation is the occurrence of gene exchange between ostensibly unrelated microorganisms. Evidence is gradually accumulating in support of promiscuous transmission of genetic material, especially resistance determinants and virulence factors, where it was previously imagined that exchange was limited by restriction barriers (Davies, 1994). The focus of this work is intergeneric gene exchange, particularly as influenced by restriction barriers.

1.2 THE ANTIBIOTIC ERA

The introduction of antimicrobial chemotherapy into clinical medicine in the 1940s is widely regarded as one of the greatest successes in modern medicine. Antibiotics have been responsible for dramatic reductions in morbidity and mortality, and prophylactic measures have enabled previously infection-prone procedures to become routine (Felmingham, 1995). So great was the success of these drugs that they became colloquially referred to, somewhat naively, as 'magic bullets' (Koshland Jr, 1994). The initial impact of

antimicrobials has been tempered by the widespread emergence of resistance within microbial populations. Complacency amongst health professionals and researchers led to overconfidence in the ability of these 'wonder drugs', and subsequently their heavy use, not only in medicine but also as growth enhancers in animal husbandry (Berkowitz, 1995). Presently, the application of naturally occurring antibiotics is nearing exhaustion, necessitating the need for rational drug design, the technology for which is still in its infancy (Silver and Bostian,1993, Stachelhaus et al, 1995), and the search for novel drug targets (Heinemann, 1993).

Antibiotics have always had an influence on microbial ecology. These compounds are produced naturally by many species (de Lorenzo and Aguilar, 1984). However, despite perhaps billions of years of exposure to these compounds, the number of resistant bacteria in environments shared by humans remained low (Hughes and Datta, 1983). It is generally accepted that antibiotic resistance has only become a widespread phenomenon in the past 50 years (Davies, 1995). Perhaps then it is no coincidence that this period corresponds to the discovery and subsequent mass production of antibiotics by humankind; this increase in the production and distribution of antibiotics has almost certainly accelerated the evolution of bacterial antibiotic resistance (Trieu-Cuot, 1987a, Davies, 1994). An estimated 10^{10} kg of antibiotics have been released since the 1950s (Davies, 1992) and the consequences of this anthropogenic perturbation of the global environment have been severe. The deluge of antibiotics represents an enormous selection pressure to which microorganisms have responded more rapidly than was thought possible before the antibiotic age. Moreover, the discharge of xenobiotic compounds with nonspecific antimicrobial properties further contributes to the spread of antibiotic resistance, as both types of resistance are often transmitted together (Ford, 1994, Summers et al, 1993). The extent of microbial adaptation has led to ominous warnings of the 'post-antibiotic era' (Berkowitz, 1995, Koshland Jr, 1994, Travis, 1994, Collignon and Bell, 1996).

The threat posed by increasing bacterial antibiotic resistance was recognised since discovery of the first R plasmid-borne resistance in the 1950s (Davies, 1995 and references therein). Incredibly, much of this research was difficult to publish contravening as it did the belief at the time, that antibiotic resistance was non-transmissable and not a threat to clinical treatment of bacterial infections (Davies, 1995). Since then papers have been published regularly suggesting more prudent use of antibiotics (Jackson 1974). These calls seem to have been largely ignored, with no reduction in the amount of antimicrobial agents prescribed and continued use in agriculture. An astounding proposal for over-the-counter availability of antimicrobials was one recent suggestion of the Clinton administration in the United States (Wenzel and Kunin, 1994). The result has been the reemergence of bacterial diseases previously thought to have been defeated by antimicrobial chemotherapy. Hospital isolates of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, are frequently resistant to multiple antimicrobial agents (Young, 1993). The true extent of the problem is most evident in recent surveys of the resistance patterns of common nosocomial pathogens, including *Streptococcus pneumoniae, Enterococcus faecalis*, *Pseudomonas aeruginosa*, and members of the *Enterobacteriaceae* (Neu, 1992, Felmingham, 1995, Silver and Bostian, 1993, Chen et al, 1995, Ciofu et al, 1994, Collignon and Bell, 1996). In all of these surveys, both the incidence and the magnitude of resistance has increased to such an extent that there are now isolates from most genera with resistance to one or more commonly used agents.

1.3 MECHANISMS OF ANTIBIOTIC RESISTANCE

The molecular mechanisms of antibiotic resistance are as diverse as the agents themselves. An abundance of recent reviews detail the biochemical and biophysical nature of these mechanisms (Davies 1994, Amyes and Gemmell 1992, Hayward and Griffin 1994) and the mode of action of clinical antimicrobial drugs (Yao and Mollering, 1995). Although diverse, mechanisms of resistance are traditionally grouped into three main categories: enzymatic inactivation of the antibacterial agent; decreased intracellular concentration (due to changes in permeability or active efflux) reducing access to target sites; alteration of the cellular target rendering the antibiotic ineffectual (Neu, 1992).

1.4 DISSEMINATION OF RESISTANCE GENES

Organisms which synthesise antibiotics are often intrinsically susceptible to the compounds they produce (Amabile-Cuevas and Chicurel, 1992). The mechanisms by which antibiotic-producing species protect themselves against their toxic metabolites are often similar to the mechanisms employed by antibiotic resistant pathogens, suggesting that the resistance determinants in non-producing resistant strains were acquired from antibiotic producers (Amabile-Cuevas and Chicurel, 1992). Comparison of the genes from these different sources supported the hypothesis that resistance determinants in antibiotic-producing organisms are the source of resistance in clinical isolates (Trieu-Cuot et al, 1987a).

Resistance genes are also grouped based on their genetic origin. Under this classification, resistance is either endogenous or exogenous (Silver and Bostian, 1993). An endogenous resistance determinant is a recessive mutation conferring resistance in the presence of the drug which is often detrimental when the selection is removed. Exogenous resistance comprises a horizontally

transferred dominant resistance which is usually stable in the absence of the antibiotic. It is the latter class which has seen antibiotic resistance spread at such an incredible rate (Amabile-Cuevas and Chicurel, 1992).

In many cases, conjugative plasmids provide the vehicle for the horizontal transfer of genetic material, both within a species and between genera. In particular, the transfer of resistance genes is frequently mediated by R plasmids, which often harbour several resistance determinants. These plasmids are believed to have evolved rapidly from conjugative plasmids which were previously devoid of resistance determinants (Hughes and Datta, 1983). In addition to conjugation, transformation is likely to provide a prevalent route for horizontal transfer of resistance genes (Davies,1994, Lorenz and Wackernagel, 1994).

1.4.1 Conjugation

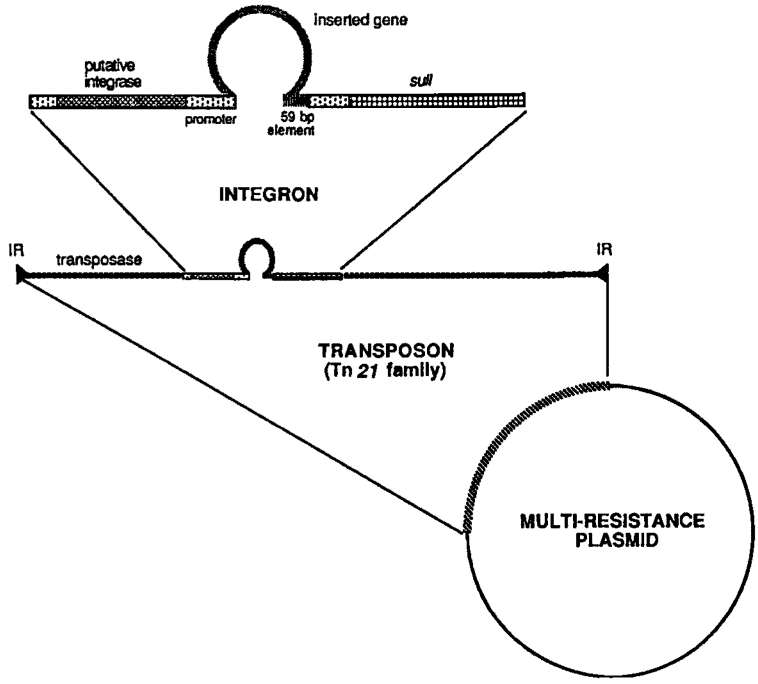
Conjugation is best described as cell contact-dependent transmission of genetic material with no exposure of the DNA to the external environment (Heinemann, 1992). Plasmid transmission occurs in 4 basic steps (Heinemann, 1992): contact between donor and recipient cell; transfer of plasmid DNA to the recipient; establishment of the plasmid in the recipient; segregation of the plasmid during cell division. There are 25 distinct families of plasmids, all with specific transfer processes (Guiney, 1993), and host ranges which may be narrow or broad. These families are termed Inc (for incompatibility) groups (Guiney, 1993), of which Inc F and Inc P are the best characterised with respect to their DNA mobilisation and transfer functions.

It was long thought that plasmid exchange was a specific process which only took place between closely related bacterial species. However, these imaginary barriers have been steadily eroded by research in the last ten years. The demonstration of IncP plasmid transfer between *Pseudomonas*, *Serratia*

and *Proteus* (Thomas and Smith, 1987) and F plasmid transfer from *E. coli* to *Pseudomonas* (Guiney, 1982) were among the first discoveries to suggest that conjugation was not recipient specific. Then, following demonstration of plasmid transmission between Gram-negative and Gram-positive bacteria (Trieu-Cuot et al, 1987b), it was shown that a plasmid could be transferred from a bacterium to a yeast cell (Heinemann, 1991). It is now suggested that conjugation is recipient-independent and that theoretically transfer from bacteria to any cell is possible (Heinemann, 1992).

Overlapping host ranges of R plasmids potentiate the rapid exchange of resistance genes between distantly related bacteria. The potential for plasmid-mediated spread of resistance genes becomes greater when it is considered that plasmid transmission is not always necessary for gene transmission. A transferred plasmid need not be maintained as long as the genes it carries are incorporated into the genome of the new host (Salyers et al, 1995). Mobile genetic elements termed integrons posses a recombinase function enabling them to incorporate antibiotic resistance gene 'cassettes' (Stokes and Hall, 1989, Collis et al, 1993) Integrons can mediate their own integration into transposons (which move between R plasmids and bacterial chromosomes) and probably bacterial replicons as well (Davies, 1994). In this way some R plasmids have accumulated genes conferring resistance to several antimicrobial agents (Figure 2, Amabile-Cuevas, 1992). This process may have been responsible for the evolution of R plasmids from conjugative plasmid precursors (Davies, 1994).

Figure 1.1. Molecular evolution of resistance plasmids. A model showing the sequential integration of genetic elements into modern resistance plasmids. The multi-resistance plasmid is the end result of this cascade. This is the important feature of the model: a plasmid which has acquired numerous resistance determinants, enabling co-transmission of multiple resistances to a new host cell. Adapted from Amabile-Cuevas and Chicurel, 1992



Conjugative transposons, which also have a broad host range may also contribute to this spread of resistance genes, both by their own transmission and by the extension of R plasmid host ranges (Salyers et al, 1995). Conjugative transposons are common in *Bacteroides* spp, which account for a large component of the gut microbiota, and this increases the exposure of potential pathogens to antibiotic resistance genes (Davies, 1994). Another factor influencing the distribution of antibiotic resistance is the occurrence of conjugation in environments which concentrate the microbiota from the gut with potential pathogens, such as raw sewage. Conjugation has been observed in situ in sewage treatment facilities (Altherr and Kasweck, 1982).

1.4.2 Transformation

DNA is taken up from the environment is taken up and assimilated into the genome of a recipient cell in a heritable form by a process called transformation (Lorenz and Wackernagel, 1994). Natural receptivity to DNA is termed genetic competence, a process that is genetically determined and involves the production of specific proteins (Lorenz and Wackernagel, 1994). The transformation mechanism is best understood in *Streptococcus pneumoniae* and *Bacillus subtilis* (Dreiseikelmann, 1994). Relatively few bacteria are naturally competent, but most genera can be induced into a physiologically competent state given the right environmental conditions. Such conditions include rapid temperature changes and local variations in electrolyte concentrations - similar to the conditions employed in artificial transformation in the laboratory. As similar conditions exist transiently in the environments naturally inhabited by microorganisms, this suggests that transformation has had a prevalent role in both the origin of antibiotic resistance genes and their spread (Davies, 1994, Lorenz and Wackernagel, 1994).

Reports suggest that many of the genes acquired by resistant pathogens originate in the very organisms that produce antibiotics (Webb and Davies, 1993). Both the mechanism of resistance and molecular characterisation of the genes responsible support this idea (Bissonnette and Roy, 1992, Davies, 1992). Further evidence was provided by studies which not only demonstrated that DNA can be isolated from commercial antibiotic, but also that this DNA was capable of transforming previously sensitive bacteria (Chakrabarty et al, 1990). The isolated DNA was shown to be an impurity in the isolated antibiotic extracted from batch cultures of the producing organism (Webb and Davies, 1993).

Free DNA of high molecular weight has so far been detected in all environmental sites tested, specifically sea water, fresh water and sediments

(DeFlaun et al, 1986). That DNA present in the environment can be responsible for transformation is at first surprising since DNases are ubiquitous and cause rapid degradation of naked DNA molecules (Lorenz and Wackernagel, 1994). However, DNA persists due to both the sorptive properties of minerals and organic polymeric compounds which rapidly sequester DNA and prevent its enzymatic degradation, and the continual release of DNA by microorganisms (Lorenz and Wackernagel, 1994). In addition to these environments, studies have also demonstrated the resistance of DNA to digestion in the gastrointestinal tract (Schubbert et al, 1995) and in the muscle tissue of mice (Ulmer et al, 1993). Thus it is clear that the necessary conditions for the extracellular persistence of DNA, and the ability for bacteria to uptake free DNA (both naturally and induced), are present in a variety of microbial habitats.

1.5 RESTRICTION BARRIERS TO GENETIC EXCHANGE

Restriction was the term coined to describe the different efficiencies of phage plating on different host strains (Heitman, 1991). Although these differences were later attributed to restriction-modification enzyme systems, the term originally described the general phenomenon of reduced DNA transmission. For genes to be effectively transmitted during horizontal transfer, there are two barriers which must be overcome (Heinemann, 1991, Matic et al, 1996): the barrier preventing gene delivery and the establishment barrier preventing inheritance. Environmental stresses can cause changes in the efficacy of restriction barriers. The nature of these changes depends on the stress and the species involved.

The discovery of restriction enzymes and their use in modern molecular genetics has seen them become the main focus of research concerned with restriction barriers. Consequently, research on the establishment barrier has

continued with less emphasis on barriers to delivery. Despite this, it is known that the cell surface has an integral role in gene transmission through its involvement in the DNA uptake step of all modes of gene transfer.

1.6 INVOLVEMENT OF THE CELL ENVELOPE IN GENE TRANSMISSION AND RESISTANCE

1.6.1 Membrane Dynamics

The cell wall and outer membrane of gram-negative bacteria provide a semi-permeable barrier between the cytoplasm and the external media, and tightly regulate the uptake of molecules into the cell. The outer membrane is organised as an asymmetric bilayer, with lipopolysaccharide (LPS) molecules arranged in the external layer and phospholipids comprising the inner layer (Nikaido and Vaara, 1985). The outer membrane is in direct contact with the peptidoglycan component of the cell wall through strong non-covalent and covalent interactions (Hancock and Bell, 1988). The segregation of LPS and phospholipids creates a membrane which is impermeable to hydrophobic substances, protecting enteric bacteria in particular from detergents in the gastrointestinal tract (Nikaido and Vaara, 1985). Anchored within the outer membrane a variety of proteins are which are involved in both general and specific diffusion processes (Nikaido and Vaara, 1985).

Small hydrophilic molecules can traverse the outer membrane via protein channels called porins, but only when small enough to pass through the dimensions of the protein pore. Hydrophobic compounds are able to diffuse through the LPS of the outer membrane to a limited extent; the lack of glycerophospholipids in the outer surface bars efficient diffusion (Vaara, 1992). Electrostatic cross-linking of LPS molecules by divalent cations such as Mg^{2+}

further increases the stability of the outer membrane, enhancing its barrier function (Nikaido and Vaara, 1985).

1.6.2 Involvement in Resistance Mechanisms

The barrier function of the gram-negative outer membrane confers intrinsic resistance to antibiotics effective against other bacteria (Nikaido and Vaara, 1985). As most antibiotics are large hydrophilic or hydrophobic compounds this feature either prevents or restricts diffusion of antibiotics into the cell (Vaara, 1992). When reduced uptake is combined with enzymatic-inactivation of the antibiotic high level antibiotic resistance is easily attained (Nikaido and Vaara, 1985).

Hydrophobic antibiotics which can cross the outer membrane, such as aminoglycosides, do so by disrupting the integrity of the lipid bilayer (Hancock and Bell, 1988). The polycationic nature of aminoglycosides enables them to displace the divalent cations in the outer membrane which are essential for LPS-stabilising interactions (Nicas and Hancock, 1980). Divalent cation displacement and subsequent interactions between aminoglycosides and LPS molecules permeabilises the outer membrane resulting in increased aminoglycoside uptake (Hancock and Bell, 1988). This process is known as the self-promoted uptake pathway and is thought to occur in most gram-negative bacteria (Hancock and Bell, 1988).

1.6.3 Transport of DNA across bacterial membranes

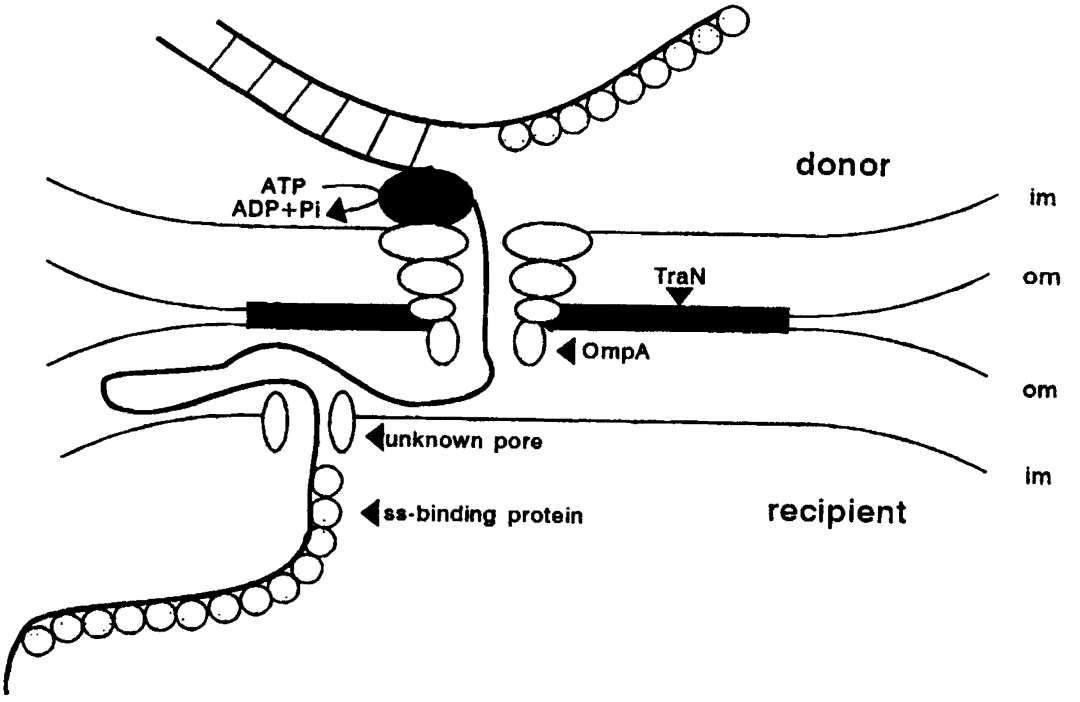
1.6.3.1 conjugation

The plasmid-encoded functions for DNA mobilisation and transfer have been extensively characterised for Inc F and Inc P plasmids. However, the specific route for transport of plasmid DNA from donor to recipient has yet to be elucidated. Little is understood regarding the membrane structures involved, and this is compounded by the apparent lack of conjugation-specific proteins in the recipient (Heinemann, 1992). Originally, DNA was thought to be translocated via the pilus, an organelle produced by all cells containing a conjugal plasmid (Dreiseikelmann, 1994). Despite being necessary for conjugation and having dimensions sufficient for the passage of a DNA molecule, evidence for a pore function is inconclusive (Heinemann, 1992). Another popular theory involves DNA transport through a pore formed by fusion of the cytoplasmic and outer membranes of the donor and recipient (Willetts and Wilkins, 1984), but this is not visualised under electron microscopy (Sabelnikov, 1994).

The formation of a protein or membrane pore during the conjugation event has yet to be visualised despite numerous studies. The most recent evidence suggests an involvement for membrane fusion (Sabelnikov, 1994). Observations implicating outer-membrane fusions are the most commonly reported, with cytoplasmic membrane fusions seen only infrequently (Sabelnikov, 1994). A currently favoured model (see figure 1.1) suggests that DNA leaves the recipient though a pore and enters the periplasm of the recipient through an outer membrane fusion (Durrenberger, 1991). TraN may be involved in pore formation, as it is known to stabilise the mating pair (Manneewannakul et al, 1992). From here it is translocated across the cytoplasmic membrane via a pore of unknown composition, perhaps the mannose uptake system involved in the uptake of bacteriophage λ DNA

(Scandella and Arber, 1976), or passive transmembrane ion channels (Singleton, 1983).

Figure 1.1. The conjugation junction. This model by Durrenberger et al (1991) incorporates the current theories concerning roles for proteins involved in conjugation. The black ellipse with ATPase function is TraC. Despite the apparent completeness, several roles are assigned based on indirect observations.



1.6.3.2 transformation

In naturally competent bacteria, transformation is a genetically and physiologically controlled process. Specific membrane-bound proteins are produced which interact with DNA and escort it into the cell, circumventing the physical barrier imposed by the outer membrane. In non-competent species, the outer membrane provides a reliable barrier to DNA uptake, preventing transformation. However, bacteria which are not naturally competent can be induced to take up nucleic acids under laboratory conditions. This phenomenon has been particularly valuable in molecular biology, with

techniques for inducing DNA uptake routinely used. The best characterised of these treatments are cation-induced uptake and electro-stimulated uptake.

Induced DNA transfer is the least specific and least efficient process for DNA transfer. A vast excess of DNA is required and only a small proportion is successfully translocated into the cell. Cation-induced uptake involves nonspecific permeabilisation of the outer membrane followed by DNA adsorption onto the cell surface, and finally a heat shock step which provides the impetus for DNA translocation (Sabelnikov et al, 1975). Cell aggregation is also necessary for the interaction with DNA, and is stimulated by high concentrations of divalent cations (Borovjagin et al, 1987). Treatment with divalent cations also enriches the outer membrane with phospholipids and in conjunction with the requirement for heat, may implicate an important role for phospholipids (Sabelnikov, 1994). Concentration of phospholipids in localised areas (Cullis and de Kruijff, 1979) and polymorphic membrane changes, such as nonbilayer lipid phase formation causing nonspecific outer membrane permeability (Borovjagin et al, 1987), have also been observed under conditions of maximal DNA transfer. It is not unreasonable to suggest that polymorphic changes in bacterial membranes may occur during favourable fluctuations in a cell's local environment, and that this may potentiate genetic exchange (Borovjagin et al, 1987).

1.7 AIMS OF PRESENT RESEARCH

In this research it was hoped to address the role of the cell surface as a barrier to genetic exchange and the effect of heat, antibiotics and other stress on the functioning of this barrier. The systems employed to test this were interspecific conjugation between *E. coli* and *S. typhimurium*, and cell fusion between ampicillin-treated *E. coli* strains. Chapter II details the experiments carried out during characterisation of the restriction barrier in *S. typhimurium*, and Chapter III outlines the experiments involved in the investigation of *E. coli* fusion.

CHAPTER II

THE EFFECTS OF ELEVATED TEMPERATURES ON RESTRICTION BARRIERS

1. BACKGROUND.

As outlined in Chapter I, genetic transmission is limited by restriction barriers. These barriers reduce the transfer and/or stable inheritance of DNA. In most circumstances these restriction barriers may help maintain the integrity of a species by preventing either uptake or recombination of the incoming DNA molecule. However, there are conditions under which these restriction barriers are relaxed, thus potentially enabling an organism to inherit genes from an unrelated species. Some of these conditions are described below.

1.1. Temporary loss of restriction following heat shock.

Heat shock-induced alleviation of the restriction barrier in a recipient prior to DNA transfer has been demonstrated for several species using a variety of assays(Mojica-a and Middleton, 1971, Holloway, 1965, Engel, 1987, Rella et al, 1989, Schafer et al, 1990). Mojica-a and Middleton (1971) noted that preincubation of *S. typhimurium* LT2 at 50°[1] led to a 1000-fold increase in prototrophic recombinants recovered after mating with Hfr *E. coli* strains. The pair also demonstrated that the change in restriction was temporary; once returned to 37°, the heat-shocked cells reasserted their restriction barrier within 50 minutes.

[1]All temperatures are °C but for clarity only the degree symbol is used

Heat-reduced restriction in *P. aeruginosa* displays different phenomenology. For a change in the *Pseudomonas* restriction phenotype to be observed, the recipient must grow for at least five generations at 43° (Holloway, 1965). Restriction remains inhibited for 60 generations during growth at 37° (Holloway, 1965). Loss of restriction after exposure to elevated temperatures in other recipient species has usually been demonstrated using a short incubation at the elevated temperature followed by a return to the optimal growth temperature for the transformation or conjugation procedure (Manson and Yanofsky, 1976, Dahl and Manson, 1985).

1.2. Other physical perturbations of the restriction barrier.

In addition to the effect of heat on the restriction barrier, other stresses can induce conjugal competence in *Corynebacterium glutamicum* (Schafer et al, 1994a). Exposure to organic solvents such as ethanol, shifts in pH or detergents all resulted in increased fertility of *C. glutamicum* in interspecific matings with *E. coli* (Schafer et al, 1994). Both ethanol and elevated temperatures denature protein, but in addition ethanol can cause changes in the composition of membranes (Sullivan et al, 1979). Detergents such as sodium dodecyl sulfate (SDS) also denature proteins and react with the cell wall (Adamowicz et al, 1991). It has been suggested that stress sensitivity may be a general feature of restriction systems, conferring an advantage on environmentally stressed cells by enabling them to more easily acquire genes that adapt them to the stress (Schafer et al, 1994).

In this study, the effect of temperature on genetic transmission via conjugation was investigated. Although this phenomenon has been investigated before, several aspects justify continued work. In particular, the involvement of the cell envelope in conjugation is recognised but still poorly characterised. This chapter details experiments designed to characterise the barriers to genetic

exchange that exist in *S. typhimurium*, with respect to the involvement of the cell envelope.

2. MATERIALS AND METHODS.

2.1. BACTERIAL STRAINS AND PLASMIDS.

The bacterial strains and plasmids used in this study are listed in Table 1.

Table 2.1. Bacterial strains and plasmids.

Strain or Plasmid	Relevant Genotype/Plasmid	Reference/Source
E. coli		
JB566	*hsdS* Δ(*srlR-recA*)306::Tn10 / pRS2405	J. Heinemann collection
JB570	*hsdS*, spontaneous Rf^r	J. Heinemann collection
JB436	*hsdS*, spontaneous Nx^r	J. Heinemann collection
RR1	*rpsL*20	G. Sprague collection
CS1834	VJS803 *rpsL*31	J. Klena collection
CS2429	VJS803 *cps*::Tn10 *rfaC*::Tn*lacZ rpsL*31	J. Klena collection
JB766	*hsdS* Δ(*srlR-recA*)306::Tn10 / R751	This study
S. typhimurium		
JB643	LT2 wild type spontaneous Sm^r	Roantree et al (1977)
JB644	LT2 wild type, spontaneous Rf^r	Roantree et al (1977)
JB647	LT2 *rfaC*630	Chatterjee et al (1976)
CS1857	*hsdSA*29 *hsdL*T6 *rpsL*120	DeFranco and Koshland (1981)
Plasmids		
pRS2405	IncF Ap^r	Ray et al (1986)
R751	IncP Ap^r	Taylor et al (1983)

2.2. SOLUTIONS AND MEDIA.

All media and solutions used in this study were prepared as described in Appendices 1 and 2, respectively. The antibiotics and concentrations used are listed in Appendix III.

2.3. BACTERIOLOGICAL METHODS.

2.3.1. General bacteriological methods.

All *E. coli* and *S. typhimurium* strains listed in Table 1 were grown routinely at 37°, unless specified otherwise in the text. Strains were maintained on LBH agar plates supplemented with antibiotics for daily use and these plates were stored up to one week at 4°. For long term storage, strains were stored in a mixture of LBH plus 20% glycerol at -80°. Overnight cultures were inoculated from single colonies present on selective plates and were grown under the appropriate selection. Before dilution of overnight cultures, cells were harvested by centrifugation at 8300 x g for 2 minutes and resuspended in fresh media. This washing process was repeated to ensure removal of antibiotics present in the overnight culture.

2.3.2. Mating protocol.

Conjugants were grown to saturation under appropriate antibiotic selection. Cultures were then diluted 100x, incubated with shaking and harvested in the exponential growth phase. Conjugants were then mixed in a 1:10 donor to recipient ratio with matings carried out either on a solid or in a liquid medium. All experiments had duplicates. The mating mix was incubated for 1 hour at the desired temperature. At the conclusion of the mating period, the mating mix was serially diluted in TNB. The dilution series was plated on LBH agar plates

supplemented with antibiotics appropriate for the selection and enumeration of donor, recipient and transconjugant cells, respectively. The cell titres were used to calculate the conjugation frequency per limiting parent per hour. If no transconjugants were recovered, the result was expressed as ≤1/ limiting parent titre.

2.3.2.1 Solid mating.

Solid matings were carried out on very dry LBH plates. The mating mix consisted of 50 μL of donor cells and 500 μL of recipient cells. Conjugants were combined on the plate and spread until dry, and then incubated at the required temperature for 1 hour. Following the mating period, cells were recovered in a 1 mL TNB wash across the surface of the plate.

2.3.2.2 Liquid mating.

Liquid matings were carried out in McCartney bottles for 1 hour. The mating mix consisted of approximately 5 x 10^6 donor cells and approximately 5 x 10^7 recipient cells diluted to 2 mL with fresh LBH. Following incubation in a static incubator at the prescribed temperature, the mating mix was agitated briefly with an auto vortex mixer and diluted for enumeration.

2.3.2.3. Matings in the presence of antibiotics.

Experiments conducted in the presence of an antibiotic for the duration of the mating period were performed in liquid media as above with relevant alterations. Where appropriate, the antibiotic was added to the recipient culture 30 minutes before mating commenced. For these matings, conjugants were mixed and diluted to 2 mL with fresh prewarmed LBH supplemented with the antibiotic at the appropriate final concentration and incubated for 1 hour. At the completion of the mating period, the antibiotic was removed by pelleting and resuspending the cells in a fresh medium, before dilution and enumeration of cells.

2.3.3. Heat shock treatment.

Where noted, recipient cultures were heat shocked before mating. A 15 minute heat shock at 50° was found to be optimal; longer heat shocks reduced recipient viability. Thus the duration heat shock in relevant experiments was 15 minutes. The heat-treated recipients were then maintained at 37° until required as recipients in the mating protocol described.

2.3.4. Replica plating.

For matings involving the plasmid pRS2405, transconjugant titre plates were often overwhelmed by satellite colonies, a common occurrence when ampicillin was used to select for plasmid transmission. In order to differentiate between 'true' transconjugants and satellite colonies, colonies formed on these initial plates were routinely transferred by replica plating to fresh media of identical composition. The replica plates were then incubated at 37° overnight, and the master plates were left at room temperature to allow colonies to regenerate. The master and replica plates were then compared to determine the true number of transconjugants.

2.3.5 $CaCl_2$ treatment of recipient cells.

Where indicated in text, recipients were treated with 0.1M $CaCl_2$ solution prior to mating. An overnight culture of the recipient was diluted 1:100 and incubated at 37° until it reached an OD_{600} of 0.5. After harvesting by centrifugation at 4000x g, the cells were resuspended in 1/5 volume of ice cold 0.1M $CaCl_2$ and incubated at 0° for 10 minutes. The cells were again harvested by centrifugation at 4000x g, resuspended in 1/10 volume of 0.1M $CaCl_2$ and maintained at 0° until required. Matings using cells treated in this way differed in that only 50 μL of the recipient culture was mixed with donor cells. This mixture was then diluted by the addition of 1 mL of prewarmed LBH. The mating period and other manipulations were as outlined above.

3. RESULTS.

3.1. A restriction barrier affects F plasmid conjugation between *E. coli* and *S. Typhimurium*

Conjugation between an *E. coli* donor and an *S. typhimurium* recipient is subject to a restriction barrier (Mojica-a and Middleton, 1971). This barrier significantly reduces the frequency of plasmid transmission to *S. typhimurium* compared with the frequency observed for an *E. coli* recipient. The effect of this phenomenon on interspecific transfer of pRS2405, a prototype F plasmid, was investigated. As shown in Table 3.1, the rate of F plasmid transmission from an *E. coli* donor to an *E. coli* recipient was approximately 1. In comparison, the frequency of transfer to an *S. typhimurium* recipient was, at best, $1x10^{-6}$; transconjugants commonly formed at a frequency below detection. Thus, the quantitative effect of the restriction barrier in *S. typhimurium* was a 10^6-fold reduction in transmission.

Table 3.1. Frequency of intra- and interspecific F plasmid transmission.

donor (strain)	recipient (strain)	frequency[a]
E. coli (JB566)	*E. coli* (RR1)	$1 \pm 5x10^{-2}$
E. coli (JB566)	*S. typhimurium* (JB643)	$1x10^{-6} \pm 5x10^{-7}$

[a]All frequencies are expressed as transconjugants per limiting parent per hour

3.2. The effect on conjugation of recipient growth and mating at 43°.

Elevated culture temperatures are known to reduce restriction barriers in a range of species (Mojica-a and Middleton, 1971, Schafer et al, 1990, Holloway, 1965). To enable the *E. coli* and *S. typhimurium* conjugation system to be compared to the system involving *E. coli* and *P. aeruginosa*, 43° was the temperature chosen. A stable phenotypic change occurs in *Pseudomonas* after growth at this temperature (Holloway, 1965). Table 3.2 shows the various combinations of growth and mating temperatures used.

Table 3.2 Effect of two different temperatures on conjugation.

	recipient[a]	Conditions: growth[b]/mating[c] (°C)	Frequency[d]
1	*S. typhimurium*	37 / 37	$1x10^{-6} \pm 5x10^{-7}$
2	*S. typhimurium*	37 / 43	$6x10^{-3} \pm 2x10^{-3}$
3	*S. typhimurium*	43 / 37	$1x10^{-6} \pm 4x10^{-7}$
4	*S. typhimurium*	43 / 43	$3x10^{-2} \pm 3x10^{-3}$
5	*E. coli*	37 / 37	$1 \pm 5x10^{-2}$
6	*E. coli*	37 / 43	$1 \pm 3x10^{-1}$

[a]The *S. typhimurium* recipient was JB643, the *E. coli* recipient was RR1; [b]Recipient growth temperature prior to mating; [c]Temperature at which conjugants were incubated during mating; [d]Per limiting parent

The results show that mating at 43° was the principle factor resulting in an increase in transmission frequency. The most pronounced increase in frequency (row 4) - 1000-fold - was observed when mating at 43° followed growth of the recipient at 43°. This was less than 50-fold more than the frequency obtained when the recipients were only exposed to 43° during the mating period (row 2). Surprisingly, recipient growth at 43° followed by mating at 37° produced no increase in the conjugation frequency compared with the control which received no exposure to 43°. Thus growth at 43° accentuated, but was not necessary for, increased efficiency of plasmid transmission. No residual effects of incubation at 43° were detected in recipients because the restriction barrier returned immediately at 37°.

To ensure that the increase observed in interspecific plasmid transmission following mating at 43° was not simply an artefact of elevated reaction rates, conjugation at 43° between an *E. coli* donor and an *E. coli* recipient was carried out. Increasing the mating temperature to 43° was not sufficient to change the frequency of transmission observed at 37° (see Table 3.2, rows 5 and 6). In both cases the frequency was approximately 1 transconjugant per donor.

An attempt was made to compare the results obtained above with conjugation between *E. coli* and *P. aeruginosa* at 43°. Previous studies had shown alleviation of restriction following growth at 43°, but to my knowledge mating at 43° had not been reported. The immediate difficulties presented by this system are that *P. aeruginosa* possesses high levels of intrinsic resistance to many antimicrobial agents, due to the low permeability of its outer membrane (Nakae, 1995). It was hoped to circumvent this problem by growth of the *P. aeruginosa* recipient in Mg^{2+}-limited media (Gilleland et al, 1974). This had been shown to increase the susceptibility of *P. aeruginosa* to antimicrobial agents including chloramphenicol and nalidixic acid (Young and Hancock, 1992). This increase in susceptibility makes possible the use of these drugs to discriminate between resistant strains of *E. coli*, and sensitive pseudomonads. Unfortunately, these published results could not be reproduced. Despite several attempts, a sufficient increase in susceptibility of *P. aeruginosa* to chloramphenicol and nalidixic acid was not observed (data not shown). This may have been due to the use of a different strain.

3.3. The '43° effect' is not dependent on protein synthesis.

The rapid changes in restriction observed during exposure to 43° and following a return to 37° suggested that new protein synthesis was not a requirement either for reducing or re-establishing the restriction barrier. To test this hypothesis, the mating protocol was amended by the addition of protein synthesis inhibitors at various stages, and their effect on the transmission frequency was monitored. Rifampicin or tetracycline was added either before the 37° grown-recipient was mated at 43°, or after recipient growth at 43° and prior to mating at 37°. Within minutes these agents completely inhibit RNA synthesis and protein synthesis respectively (Kingsman and Willetts, 1978). By preventing de novo protein synthesis before exposure to 43°, and after growth at 43° prior to mating at 37°, any requirement for new proteins in the absolution or reassertion of the restriction barrier would be blocked.

Table 3.3 Effect of protein synthesis inhibitors and temperature on conjugation

	recipient[a]	growth[b]/mating[c] conditions (°C)	plasmid transmission frequency[d]	
			-antibiotic	+antibiotic
1	*E. coli*	37 / 37	$5 \pm 5 \times 10^{-1}$	$2 \pm 3 \times 10^{-1}$(+Tc)
2	*E. coli*	37 / 37	$7 \times 10^{-1} \pm 5 \times 10^{-1}$	$2 \times 10^{-1} \pm 2 \times 10^{-1}$(+Rf)
3	*S. typhimurium*	43 / 37	$\leq 4 \times 10^{-6}$	$\leq 3 \times 10^{-6}$(+Tc)
4	*S. typhimurium*	37 / 43	$5 \times 10^{-3} \pm 6 \times 10^{-4}$	$3 \times 10^{-4} \pm 6 \times 10^{-5}$(+Tc)
5	*S. typhimurium*	43 / 37	$\leq 8 \times 10^{-7}$	$\leq 6 \times 10^{-6}$(+Rf)
6	*S. typhimurium*	37 / 43	$2 \times 10^{-3} \pm 7 \times 10^{-4}$	$3 \times 10^{-3} \pm 8 \times 10^{-4}$(+Rf)

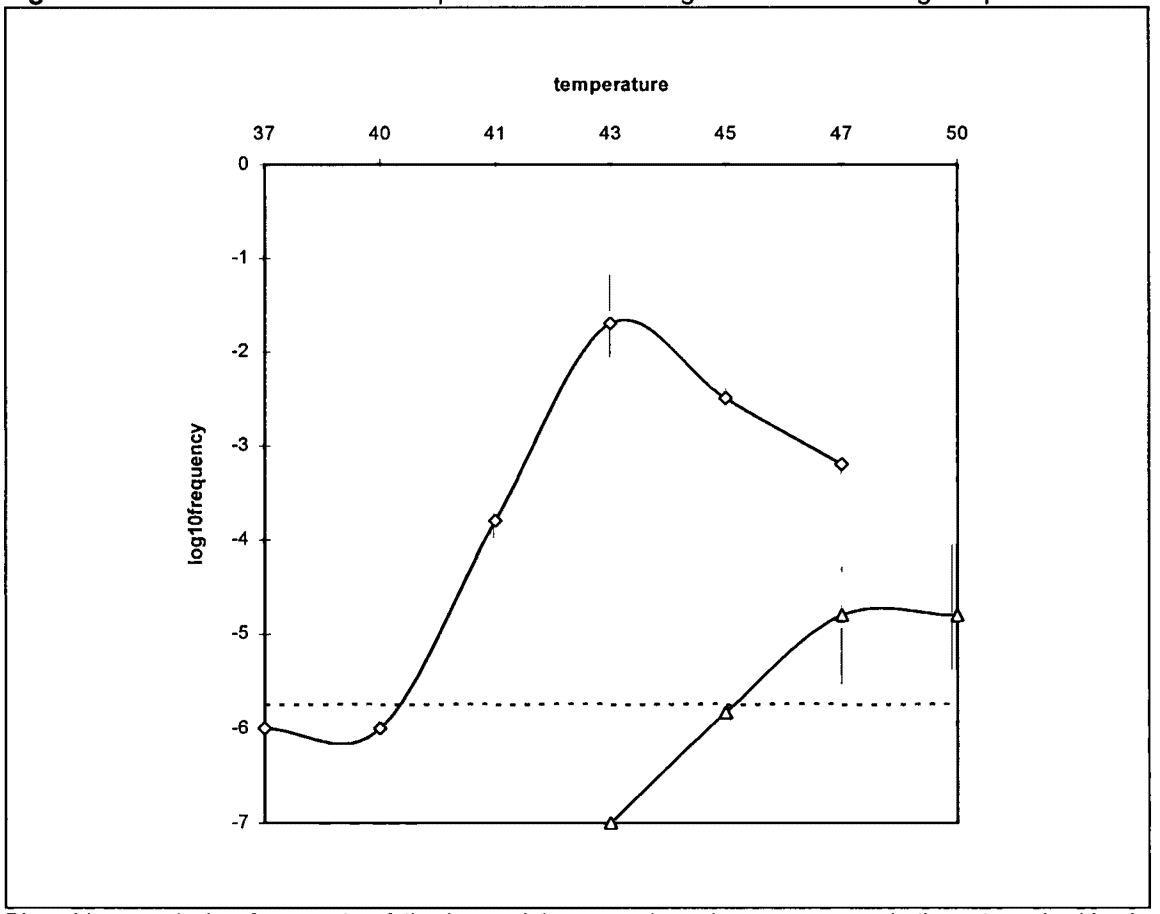
[a]The *E. coli* recipient was RR1, the *s. typhimurium* recipient was JB643; [b]Recipient growth temperature prior to mating; [c]Temperature at which conjugants were incubated during mating; [d]Frequencies are an average of 4 independent matings.

As shown in Table 3.3, in most cases the addition of protein synthesis inhibitors had no effect on conjugation involving *E. coli* donors and either *E. coli* or *S. typhimurium* recipients. Adding rifampicin before or during mating did not produce a change in the transmission frequencies observed for any mating at 37° or 43°. Tetracycline appeared to have a marginal effect on plasmid transmission from *E. coli* to *S. typhimurium* at 43°, but as this was unaffected by rifampicin, and tetracycline did not affect conjugation involving an *E. coli* recipient, this result was not considered to be significant. The results from this series of experiments confirm that new protein synthesis is required neither for the loss of restriction following an increase in growth temperature to 43°, nor for the return of the restriction barrier upon return to 37°.

3.4. Alleviation of restriction over a range of mating temperatures.

The effect of temperature on conjugation was measured over a range of mating temperatures. Figure 3.1 shows the range of temperatures tested and the change in conjugation observed respective to the control mating at 37°. Again, a combination of recipient growth and/or mating at the elevated temperature was carried out for each conjugation set. As seen in the graph, increases in plasmid transmission were observed at all temperatures with the maximum increase observed for matings carried out at 43°. Interestingly, the graph also shows that some of the higher temperatures led to a minor increase in transmission frequency when the mating temperature was returned to 37°. This phenomenon will be addressed further in sections 3.6. and 3.7.

Figure 3.1 Plasmid transmission frequencies at elevated growth and/or mating temperatures.

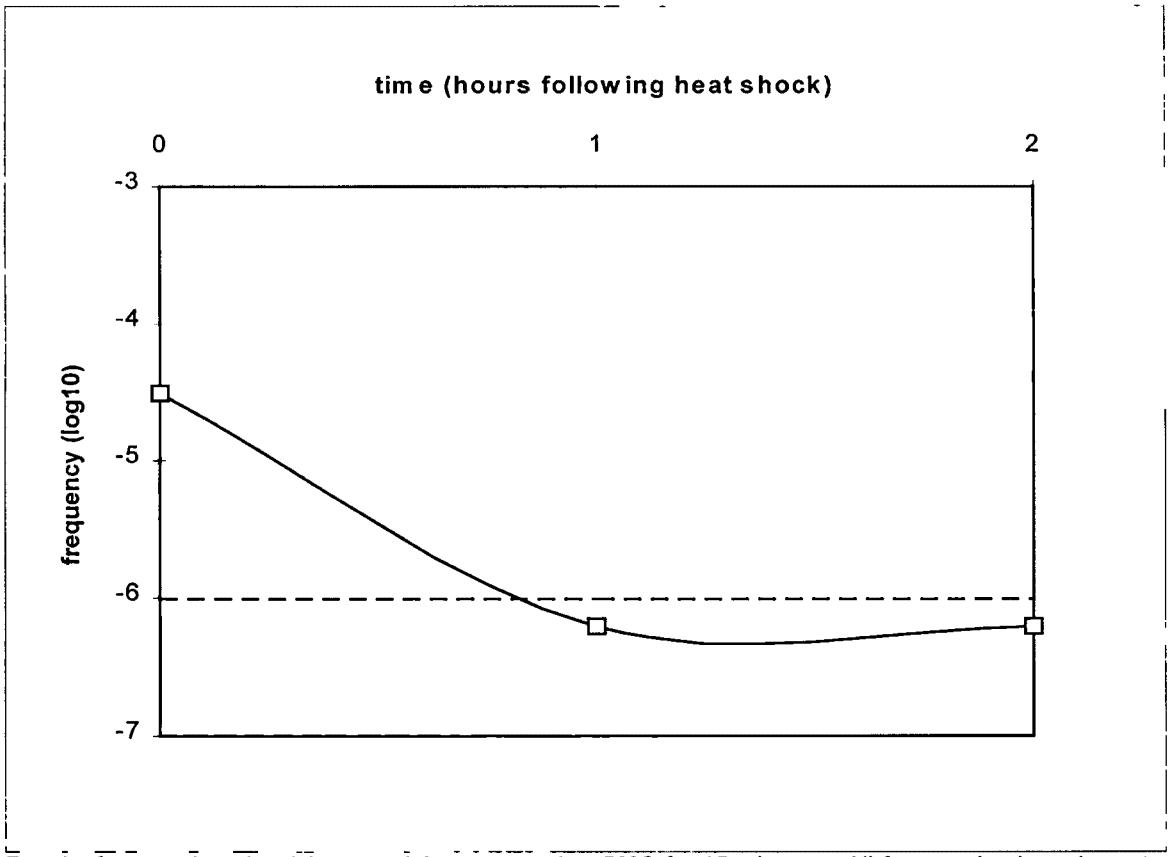


Plasmid transmission frequencies following recipient growth at the temperature indicated on the Y-axis and either mating at the same temperature (-◊-) or at 37° (-Δ-). All frequencies based on at least two independent replications. Values below the dotted line are the calculated theoretical maximum based on conjugant numbers in at least 3 crosses where no transconjugants were detected.

3.5. A 50° heat shock causes a stable change in restriction.

Previous studies demonstrated that a 50° heat shock could confer on a *S. typhimurium* recipient a change in the restriction phenotype that persisted for up to 50 minutes, sufficient to increase Hfr-mediated marker transfer (Mojica-a and Middleton, 1971). In this study, it was found that heat shock also increased the permissiveness of an *S. typhimurium* recipient with respect to F plasmid transmission. As shown in Figure 3.2, the frequency of plasmid transmission at 37° to a heat-shocked recipient increased relative to unshocked recipients.

Figure 3.2 Transmission frequencies following a 50° heat shock.

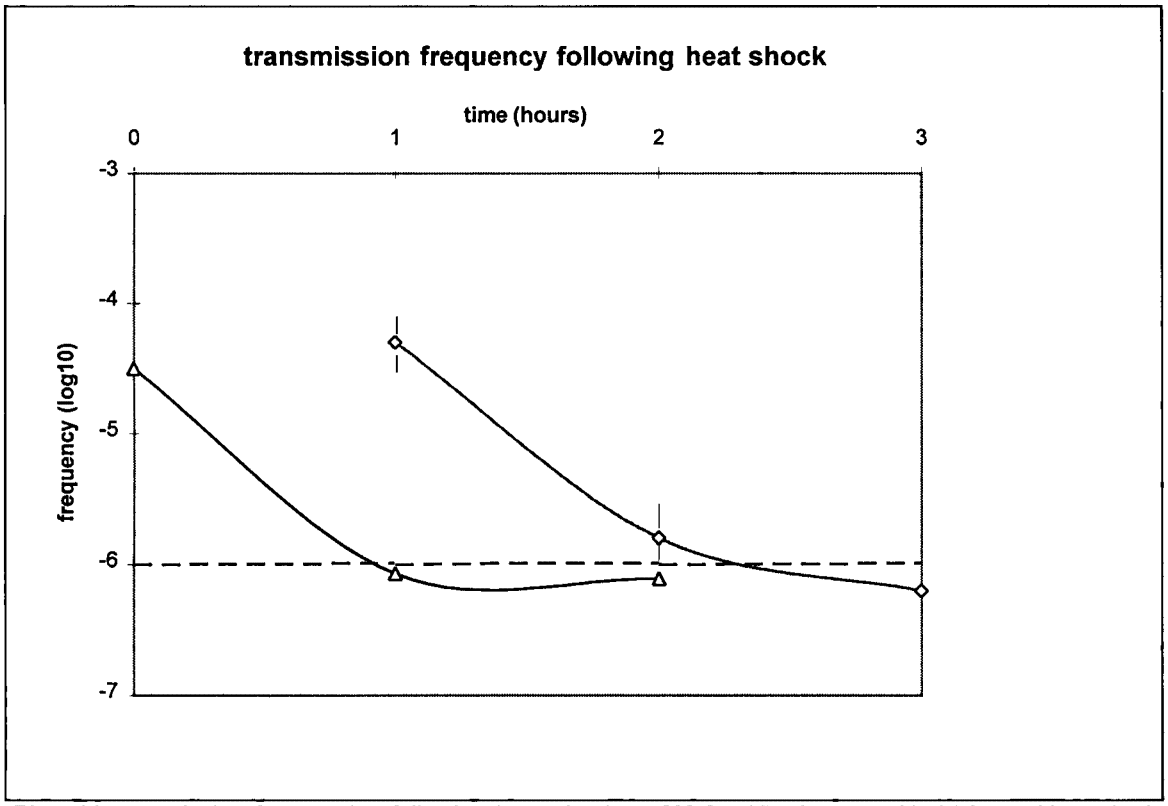


Results from matings involving a recipient exposed to 50°C for 15 minutes. All frequencies based on at least two independent replications. Values below the dotted line are the calculated theoretical maximum based on conjugant numbers in at least 3 crosses where no transconjugants were detected.

3.6. Protein synthesis is needed to reassert the restriction barrier after a 50° heat shock.

A second series of time course experiments incorporating a protein synthesis inhibitor was performed to determine whether the persistent loss of restriction demonstrated in section 3.6. was due to a loss of functional proteins. Following heat shock at 50°, the recipient cells were exposed to tetracycline for 1 hour to prevent de novo gene expression after heat shock. The antibiotic was then removed and the cells were used as recipients. Figure 3.3 shows that the 1 hour treatment with tetracycline delayed the return of the restriction barrier; once the antibiotic was removed the restriction barrier was reasserted during growth at 37° for one hour.

Figure 3.3 Tetracycline-delayed reassertion of the restriction barrier.



Plasmid transmission frequencies following heat shock at 50° for 15 minutes, with (-◊-) or without (-Δ-) exposure to Tc for 1 hour. All frequencies based on at least two independent replications. Values below the dotted line are the calculated theoretical maximum based on conjugant numbers in at least 3 crosses where no transconjugants were detected.

3.7. Mating at 43° reverses the poor recipient ability of an *rfaC* mutant.

The *rfaC* gene product is involved in an early stage of LPS biosynthesis in enteric species (Schnaitman and Klena, 1993). Mutations of the *rfaC* gene compound the poor recipient ability of *S. typhimurium*, and also reduce the recipient ability of *E. coli* (Heinemann, pers. comm.). This phenotype is responsible for a 10^3-fold reduction in plasmid transmission when compared with a wild-type enteric recipient (Heinemann, unpublished observation). As no evidence was found for the involvement of a protein in the increase of plasmid transmission observed at 43°, and the phenomenon reversed rapidly upon return to 37°, it was reasoned that the membrane may have an important role. To this end, the effect of elevated mating temperature on conjugation involving *rfaC* recipients was investigated.

Table 3.4 Conjugation with *rfaC* recipients.

	recipient[a]	conditions (°C) growth[b]/mating[c]	plasmid transmission frequency[d]
1	*S. typhimurium*	37 / 37	$\leq 1x10^{-6}$
2	*S. typhimurium (rfaC)*	37 / 37	$\leq 1x10^{-6}$
3	*S. typhimurium*	37 / 43	$3x10^{-3} \pm 3x10^{-4}$
4	*S. typhimurium (rfaC)*	37 / 43	$1x10^{-3} \pm 5x10^{-4}$
5	*S. typhimurium (rfaC)*	43 / 43	$2x10^{-2} \pm 1x10^{-2}$
6	*E. coli*	37 / 37	$1 \pm 1x10^{-1}$
7	*E. coli (rfaC)*	37 / 37	$1x10^{-2} \pm 5x10^{-3}$
8	*E. coli (rfaC)*	37 / 43	$6x10^{-3} \pm 9x10^{-4}$
9	*E. coli (rfaC)*	43 / 43	$8x10^{-3} \pm 6x10^{-3}$

[a]The *S. typhimurium* recipients were JB643 (rows 1 and 3) and JB647 (rows2, 4 and 5). *E. coli* recipients were CS1834 (row 6) and CS2429 (rows 7-9). [b]Recipient growth temperature prior to mating; [c]Temperature at which conjugants were incubated during mating; [d]Frequencies calculated using results of 4 independent matings

As seen in Table 3.4, *S. typhimurium* transconjugants were formed at a level below detection here but it was shown that transmission to the *E. coli rfaC* recipient was reduced by 10^3-fold (compare row 6 with row 7). Elevating the mating temperature reversed the effect of the *S. typhimurium rfaC* mutation. As shown in Table 3.4 (rows 4 and 5), mating at 43° enabled plasmid transmission to an *rfaC* mutant to proceed at a rate comparable to that obtained with a wild-type *S. typhimurium* recipient (row 3). This result contrasts with crosses involving an *E. coli rfaC* recipient, which were not affected by the increase in mating temperature (compare row 7 with rows 8 and 9). One possibility is that the *S. typhimurium* defect (a point mutation) is not as severe as the *E. coli* defect (a transposon insertion).

3.8. Conjugation frequency with an r - recipient increases at 43°.

Restriction- (r -) describes strains for which a higher than normal receptivity is observed. In many cases the underlying genetic basis of the increase in recipient ability is uncharacterised. The effect of such a phenotype on F plasmid transmission was investigated using a conjugation series incorporating an r - *S. typhimurium* recipient (see Table 3.5). As expected, the conjugation frequency at 37° was substantially greater for the r - recipient when compared with the wild-type (compare row 2 with row 1). Despite this, a consistent increase in the frequency was observed when mating took place at 43°, although the magnitude of this increase was only 10-fold (compare Table 3.5 rows 3 and 4).

Table 3.5 Effect of temperature on conjugation between *E. coli* and r+ or r- *S. typhimurium* recipients.

	Recipient[a]	mating temperature (°C)	plasmid transmission frequency
1	r +	37	$\leq 1x10^{-6}$
2	r -	37	$2x10^{-2} \pm 2x10^{-2}$
3	r +	43	$6x10^{-3} \pm 2x10^{-3}$
4	r -	43	$2x10^{-1} \pm 5x10^{-2}$

[a]The r+ recipient was JB643, the r- recipient was CS1857; [b]Frequencies calculated from at least 2 independent matings

The results show that although the r - phenotype was responsible for a marked increase in transmission frequency, the additional increase after mating at 43° suggests that this restriction system is not wholly responsible for the poor recipient ability of the wild type in interspecific crosses.

3.9. IncP plasmid transfer also increases at 43°.

To determine whether the increase in mating temperature affected plasmid transmission in general or IncF transmission in particular, the effect of temperature on the transmission of the IncP plasmid R751 was also investigated. The frequency of R751 transmission to *S. typhimurium* at 37° was approximately $1x10^{-6}$, similar to that for pRS2405 at the same temperature. When mating was carried out at 43°, the frequency of transmission increased approximately 100-fold, to $1x10^{-4}$. This result suggests that elevation of mating temperature is sufficient to relax restriction barriers to transmission of both IncF and IncP plasmids.

3.10. Conjugation involving a $CaCl_2$-treated recipient.

In attempting to further characterise the restriction phenomenon in *S. typhimurium,* a series of mating experiments were carried out involving a $CaCl_2$-treated recipient. This procedure is commonly used to induce competence in cells which are not naturally competent, facilitating transformation with plasmid DNA. The process causes nonspecific damage to the cell envelope, and in conjunction with a subsequent heat shock, enables translocation of DNA into the cell. The results from these experiments are presented in Table 2.6.

Table 3.6. Transmission frequency following $CaCl_2$-treatment of recipient[a]

	mating temperature (°C)	$CaCl_2$ treatment	transmission frequency[b]
1	37	x	$\leq 1x10^{-6}$
2	37	√	$5x10^{-5} \pm 8x10^{-6}$
3	43	x	$3x10^{-3} \pm 2x10^{-4}$
4	43	√	$5x10^{-4} \pm 7x10^{-5}$

[a]The recipient was JB643; [b]Frequencies calculated from at least 2 independent matings.

Mating at 37° following $CaCl_2$ treatment resulted in an increase in plasmid transmission of at least 10-fold compared with the untreated control (rows 1 and 2). When the mating temperature was increased to 43°, an additional 10-fold increase in the transmission frequency was observed (row 3). From these results it can be seen that exposure of the recipient to a high concentration of divalent cations prior to mating generates increased receptiveness for transmission of an F plasmid. Mating at 43° further increased the transmission rate, although not by a factor equivalent to matings where mating temperature was the only variable (row 4).

4. DISCUSSION.

4.1. The effects of temperature on interspecific conjugation.

4.1.1. F plasmid transmission at 43°.

Heat-treating a nonpermissive host can alleviate the restriction phenotype, such that plasmid transmission frequencies increase in subsequent conjugation events (Mojica-a and Middleton, 1971, Schafer et al, 1990, Holloway, 1965). The results presented here show that temperature elevation during mating is sufficient to increase the frequency of plasmid transmission from an *E. coli* donor to a *S. typhimurium* recipient. Raising the temperature to 43° during mating increased transmission 10^4-fold over matings at 37°. To my knowledge this is the first report of the effects of elevated mating temperatures in interspecific crosses (also J. Heinemann, pers. comm.). Previous studies on the effects of heat on conjugation have been concerned with either temperature-sensitive transmission, shown to be a result of failure of pilus formation (Wilkins and Willetts, 1984), mating pair formation (Walmsley, 1976), or the use of heat shock to improve mating efficiency (Manson and Yanofsky, 1976).

The observed increase in plasmid transmission when mating takes place at 43° appears qualitatively different to previous reports of the effects of heat on recipient permissiveness. In these studies a short exposure of recipients to an elevated temperature, usually 50°, improved the efficiency of plasmid transmission when mating took place at 37°. In contrast, I found that neither a short heat shock at 43°, nor growth for at least 5 generations at 43° was sufficient to improve the mating efficiency at 37°; the restriction phenotype reappeared immediately when the recipient was returned to 37°. The frequency of transmission was the same as that for the control in which the recipient was never exposed to 43°. Only when mating was carried out at 43°

was the increase in transmission observed. These observations suggest a restriction mechanism not determined by irreversible denaturation and resynthesis of restriction enzymes.

Mojica-a and Middleton observed an increase in Hfr-mediated marker transmission from *E. coli* to *S. typhimurium* after heat shocking the recipient (Mojica-a and Middleton, 1971). They also found that the restriction phenotype of the recipient returned after subsequent incubation for 50 minutes at 37°. These observations were consistent with mine using F rather than Hfr donors. It was suggested that the time delay was a function of resynthesis of restriction enzymes inactivated by exposure to 50°. Although their conclusion was consistent with the observations, I am aware of no further biochemical or genetic confirmation (also J. Heinemann, pers. comm.). Later researchers make reference only to the phenomenon's usefulness (Manson and Yanofsky, 1976, Rella et al, 1989), and assert that heat shock inactivates a temperature-sensitive restriction system possibly present in the recipient (Dahl and Manson, 1985).

The differences apparent from the observations of conjugation following heat shock with those obtained at elevated mating temperature warranted a comparison of these two phenomena. If as asserted heat shock alleviated restriction by denaturing protein, whereas mating at 43° did not involve protein denaturation, only the former effect would require protein synthesis to reassert the restriction barrier. The phenomena were compared by determining the requirement for de novo gene expression at different temperatures and times by incorporating of protein synthesis inhibitors at various stages of the mating procedure. Addition of tetracycline or rifampicin, either before recipient exposure to 43° or after exposure to 43° but prior to return to 37°, made it possible to control the re-start of protein synthesis at these stages. Addition of the antibiotic before the temperature was raised did not affect the transmission

frequency. De novo gene expression at the elevated temperature was, therefore, not a requirement for the increase in receptivity. Furthermore, addition of the antibiotic after exposure to 43° did not prolong the change in restriction evident at this temperature; returning the recipient to 37° allowed immediate reassertion of the restriction barrier and a concomitant decrease in plasmid transmission to the base level observed at 37°. These observations are consistent with the lack of a requirement for protein synthesis suggested by the rapid return of the restriction barrier at 37°.

In contrast to the results above, antibiotics had a profound effect on the reassertion of the restriction barrier following a 50° heat shock. Incubation of the recipients at 37° in the presence of tetracycline for 1 hour immediately following heat shock was sufficient to delay the return of the restriction phenotype for 1 hour. When tetracycline was removed, restriction returned after a further 1 hour of growth at 37°. This observation provides evidence for the involvement of protein synthesis in the return of restriction, presumably by resynthesis of proteins denatured at 50°. Thus the two temperature phenomena described here appear to be mechanistically distinct. Although these results confirm Mojica-a and Middleton's model (1971), they provide no additional evidence that the proteins involved have endo- or exonuclease activities per se. Only in the case of corynebacteria has the explicit connection between temperature-reduced restriction and restriction nucleases been made (Schafer et al, 1994)

4.1.2. Transmission of an IncP plasmid at 43°.

The dramatic effect of mating at 43° on F plasmid transmission was not only of interest in itself but also led to the idea that such conditions could promote transmission of plasmids of other incompatibility groups. For this reason IncP plasmid transmission was also monitored at 43°. Mating at 43° increased the transmission frequency of R751 by approximately 100-fold. This was an order

of magnitude less than the effect of 43° matings on F plasmid transmission. Despite differences in the severity of the effect, transmission of both plasmids was considerably enhanced. As the recipient in both cases was the same, the discrepancy may be due to differences in the plasmid-encoded transfer machinery.

4.1.3. Effect of mating at 43° on different recipients.

The efficacy of mating at 43° in reducing the restriction barrier in *S. typhimurium* provided the impetus for experiments to test this effect with a different recipient species. It was hoped to test the effect of mating at 43° on the restriction barriers in *P. aeruginosa*, as growth at 43° produces a prolonged change in the restriction phenotype of this organism (Holloway, 1965). The high intrinsic resistance of this organism to many routinely used antimicrobial agents, however, made it impossible to use the same agents as used in the *E. coli* and *S. typhimurium* crosses. Accordingly, an attempt was made to use the findings of Young and Hancock (1992) in order to increase the susceptibility of *P. aeruginosa* to chloramphenicol and nalidixic acid thus allowing these agents to be useful in the selection of transconjugants. Unfortunately a useful increase in susceptibility was not obtained and thus the incorporation of *P. aeruginosa* into the conjugation experiments was not possible.

4.2. Evidence for involvement of the cell envelope in the 43° effect.

The rapid reversal at 37° of the changes in *S. typhimurium* restriction associated with exposure to 43°, suggests that the underlying mechanism involves something other than restriction enzymes. The phenomenon may be related to membrane dynamics. Changes in the composition of the gram-negative envelope accompanying variation in growth temperature have been well documented (Borovjagin et al, 1987, Sullivan et al, 1979). An increase in the growth temperature results in an increase in the ratio of saturated to unsaturated fatty acids incorporated into membrane lipids, and with this comes an increase in membrane fluidity. In addition, more severe treatments such as subjecting cells to combinations of heat shock, EDTA and Ca^{2+} can lead to gross rearrangements of the asymmetric outer membrane including loss of some components. In this situation some loss of LPS and its replacement by phospholipids leads to greater fluidity and permeability of the outer membrane.

The membrane-structure aspects of DNA transfer during conjugation remain largely undetermined despite continual research (Sabelnikov, 1994). From the results obtained in this study, it is tempting to assign a role for changes in membrane integrity in the increase of plasmid transmission observed at 43°. If such changes allowed a greater influx of DNA into the recipient, this could result in the restriction enzymes of the cell becoming saturated and the consequent establishment of the plasmid. Alternatively, changes in membrane permeability may decrease the effective concentration of periplasmic nucleases, leading to an increased probability of plasmid establishment (Dreiseikelmann, 1994). The results from crosses involving the r^- recipient are intriguing in this regard. As the strain is uncharacterised with respect to the genetic changes responsible for its altered restriction phenotype, it may have a change affecting a cellular component other than a restriction enzyme system. Heat shock treatment of recipient cells did not raise the transmission frequency

to the level observed with the r $^-$ recipient (at least 100-fold less), despite a demonstrated involvement for a heat-labile protein in this effect (section 3.7). In addition, incubation of the r $^-$ recipient at 43° further increased plasmid transmission by 10-fold compared with mating at 37°. Furthermore, Ca^{2+} treatment of the recipient before conjugation also resulted in an increase in the recovery of transconjugants. Treatment of this nature is known to cause changes in the cell envelope, and is responsible for inducing competence in artificial transformation. The complementarity of these known cell wall destabilising treatments and temperature on plasmid transmission suggest an involvement for the membrane in the 43° effect.

A cell with an *rfaC* mutation has a severe defect in LPS synthesis, resulting in increased permeability of its outer membrane. This mutation further reduces the frequency of plasmid transmission to a *S. typhimurium* recipient by 10^3-fold compared to a wild-type control. Surprisingly, the elevated mating temperature was sufficient to increase the receptivity of the *rfaC* mutant to a level equivalent to that obtained with a wild type recipient at 43°. As the *rfaC* mutation alters the composition of the outer membrane, it was thought that this phenotype may have a major involvement in the poor receptivity of the cell. However, the number of transconjugants formed from crosses involving an *E. coli rfaC* mutant could not be increased by mating at 43°, suggesting that either the influence of the *rfaC* mutation is different in these two species, or the difference is allele-specific.

4.3. Future work.

Further investigation is required to identify the underlying genetic and biochemical bases for the 43° effect. It would be of interest to determine whether changes in membrane integrity do indeed have an effect on mating at elevated temperature as suggested by the observations detailed here. Isolation

of a mutant which has lost its temperature-sensitive restriction phenotype, could be possible by mating *S. typhimurium* recipients to an *E. coli* donor carrying a plasmid with a lethal gene. Recipients which did not mate with increased frequency at 43°, due to loss of the temperature-sensitive phenotype, could be isolated and further characterised.

The results reported here may prove useful in future studies requiring genetic transfer to species which are generally difficult to transform. Applying this technique may abrogate the need for heat shock treatment prior to conjugation. The validity of this suggestion could perhaps be tested using an auxotrophic *P. aeruginosa* recipient and selection for prototrophy rather than antibiotic resistance. This would also address the question of whether the increase in plasmid transmission frequency at 43° is a property of recipients other than *S. typhimurium*.

CHAPTER III

THE INVOLVEMENT OF ANTIMICROBIAL AGENTS IN GENETIC EXCHANGE

1. BACKGROUND.

A variety of antimicrobial agents are active against the bacterial cell surface. As well as β-lactams which act directly on the cell wall, other compounds are also cell-surface active. Although the outer membrane is not the target for lethality, these agents disrupt the integrity of the outer membrane during translocation (Nikaido and Vaara, 1985), thereby increasing the permeability of the membrane and promoting their own uptake (Vaara, 1992). As the cell envelope is involved in genetic exchange, often limiting DNA transfer, any perturbations of this barrier may potentiate genetic transmission between unrelated organisms.

1.1. Cell fusion.

A method for DNA transfer between cells which are not amenable to transformation by other commonly used techniques involves controlled fusion of the spheroplast form of the cells. As well as occurring naturally during phenomena such as mating in yeast (Heinemann and Sprague, 1991), this process has been applied during genetic studies of cells as diverse as erythrocytes, fungi, and algae (Reina et al, 1993). The methodology involves induced formation of a spheroplast form which is then exposed to a fusogenic

agent. Until recently, natural exchange of genetic information between bacteria in this manner had been shown to occur in gram-positive bacteria only (Fodor and Afoldi, 1979). Gram-negative bacteria seemed unable to undergo this type of fusion with reliable efficiency, possibly because the outer membrane protected the cell wall from degradation and thus limited the area of cytoplasmic membrane available for fusion (Schaeffer et al, 1976). A recent study demonstrated genetic recombination by fusion of *E. coli* spheroplasts (Reina et al, 1993). This research employed sub-inhibitory concentrations of polymyxin B, which weakens the outer membrane, to improve spheroplast formation and concomitantly the fusion efficiency. The transmission of plasmids from *E. coli* to gram-positive bacteria by conjugation is also enhanced following exposure of the recipient to sub-inhibitory penicillin concentrations prior to mating (Trieu-Cuot et al, 1993).

1.2. Production of DNA-containing membrane vesicles.

A related phenomenon with perhaps a greater potential influence on genetic exchange is the production of small membrane vesicles (MVs) by many gram-negative pathogens (Kadurugamuwa and Beveridge, 1995). These were first discovered due to their involvement in transformation in *Neisseria gonorrhoeae* (Dorward et al, 1989) and are now known to be produced by at least 14 gram-negative strains (Dorward and Garron, 1990). MVs can mediate exchange of plasmid DNA between *N. gonorrhoeae* cells (Dorward et al, 1989), and in other species they contain virulence factors (Kadurugamuwa and Beveridge, 1996). In *P. aeruginosa* the production of these vesicles and their DNA content increases after exposure to gentamicin (Kadurugamuwa and Beveridge, 1995). Although DNA contained within MVs is in a closed circular (and therefore stable) form it has yet to be demonstrated whether this DNA can transform species other than *N. gonorrhoeae* (Kadurugamuwa and Beveridge, 1995).

1.3. Experimental design.

In this study it was hoped to test the idea that antibiotics may potentiate the exchange of genetic material through their effects on the cell surface. Two *E. coli* strains containing different non self-transferable plasmids were constructed and employed in experiments designed to test whether ampicillin could potentiate exchange of the plasmids between these strains via cell fusion. Unfortunately the time constraints inherent in the present study prevented the experiments and ideas from being fully explored. Nevertheless, preliminary results are presented here as a basis for future studies.

As mentioned in Chapter I, commercial preparations of antibiotics contain quantities of DNA, including resistant determinants, derived from the antibiotic-producing organism (Webb and Davies, 1993). This DNA can transform susceptible bacteria, resulting in resistance to the antibiotic from which the DNA is derived (Chakrabarty et al, 1990). It was reasoned that if the antibiotic itself causes a change in the target bacteria leaving them more amenable to fusion, then this may potentiate the transmission of the resistance determinant applied accidentally in conjunction with antibiotic therapy. Treating cells with ampicillin can induce spheroplast formation, due to the disruption of the peptidoglycan layer which gives the cell its shape and osmo-stability (Marvin and Witholt, 1987). By combining this phenomenon with observations from a recent study in which genetic exchange between *E. coli* via fusion of the spheroplast form was demonstrated (Reina et al, 1993), it was hoped to determine whether the ampicillin-induced spheroplast form conferred upon the cell a greater propensity for fusion and hence genetic exchange.

Previously demonstrated fusion involving *E. coli* has relied upon lysozyme treatment prior to the fusion event; this is true both for fusion between two *E. coli* cells (Reina et al, 1993) and fusion between *E. coli* and yeast (Sherman et

al, 1983). Following exposure to lysozyme, the spheroplast form is elaborated in an osmotically balanced media, before exposure to a fusogenic agent. As ampicillin also disrupts the cell wall, the possibility that treatment with this agent may replace the requirement for lysozyme in such procedures was tested. This would determine whether ampicillin could potentiate fusion and hence genetic exchange.

2. MATERIALS AND METHODS.

2.1. BACTERIAL STRAINS AND PLASMIDS.

The bacterial strains and plasmids used in this study are listed in Table 3.1.

Table 3.1. Bacterial Strains and Plasmids.

Strain or Plasmid	Genotype/Plasmid	Reference
E. coli		
JB570	*hsdS*, spontaneous Rf^r	J. Heinemann collection
JB436	*hsdS*, spontaneous Nx^r	J. Heinemann collection
GR970	JB570 transformed with Jp106	this study
GR936	JB436 transformed with pUC4K	this study
Plasmids		
Jp106	pACYC184 derivative, Cm^r	J. Heinemann collection
pUC4K	Km^r	Pharmacia

2.2. SOLUTIONS AND MEDIA.

All media used are described in Appendix I. Solutions and buffers used are detailed in Appendix II. Antibiotics and their concentrations are listed Appendix III.

2.3 BACTERIOLOGICAL METHODS

2.3.1. Construction of strains for fusion experiments.

2.3.1.1. Extraction of plasmid DNA by alkaline lysis.

Cells from 1.5 mL to 100 mL of an overnight culture grown in LBH were harvested by centrifugation. The pellet was resuspended in 0.1 volumes of solution I by mechanical agitation using an auto vortex mixer. Solution II was added at 0.2 volumes of the original culture volume and mixed gently by rolling of the centrifuge tube. Once lysis had occurred (solution went clear and viscous), 0.15 volumes of solution III were added and mixed until a white precipitate had formed and the solution was no longer viscous. The precipitate was then collected by centrifugation (10 minutes, 14800 x g, room temperature) and the supernatant transferred to a new tube. DNA was precipitated with 2 volumes of ice cold 100% ethanol, collected by centrifugation, washed with 1 mL of 70% ethanol and air dried before being dissolved in 20 μL of dH_2O.

2.3.1.2. Electrotransformation.

Electrocompetent cells of strains JB436 and JB570 were prepared and electroporated as described previously (Dower et al, 1988). Electroporation was carried out using a Gene Pulser™ (Bio-Rad) set with capacitance at 25 μF, pulse-controlled resistance at 200 Ω, and voltage at 1.8 kV using a cuvette with a 0.1cm gap. Transformants were elaborated by growth for 1 hour at 37° and were recovered on LBH agar plates supplemented with the appropriate antibiotics. The presence of the plasmids was confirmed by gel electrophoresis (data not shown).

2.3.2. Fusion protocols.

Two fusion protocols were adapted for use in this study: the *E. coli* protoplast transformation method of Sherman et al (1983), and the glycine and lysozyme-EDTA protocol of Reina et al (1993).

2.3.2.1. *E. coli* protoplast transformation.

Fusion partner strains were grown to saturation in 10 mL LBH and harvested by centrifugation (10 minutes at 600 x g). The cell pellets were resuspended in 500μL SE, followed by addition of 50 μL of 10 mg/mL lysozyme solution and incubation on ice for 10 minutes. The cells were again harvested by centrifugation, then resuspended in 100 μL STC. Suspensions of each strain were mixed and incubated at room temperature for 10 minutes, at which time 1.8 mL of fusion mixture was added. After 10 minutes incubation at room temperature cells were collected by centrifugation at 600 x g. The resultant pellet was resuspended in 150 μL SOS and incubated at 37° for 20 minutes. Following growth of the fusion mixtures to saturation in 10 mL S-LBH at 37°, aliquots were plated on S-LBH agar plates supplemented with the appropriate antibiotics for enumeration of parent and recombinant cells.

2.3.2.2. Glycine and lysozyme-EDTA induced spheroplast fusion.

Each fusion partner was grown to saturation in 40 mL nutrient broth. Cells were harvested via centrifugation and resuspended in 1 mL SMMD, 0.4 mL of lysozyme solution. The reaction was halted after 3 min. at room temperature by the addition of 50 μL EDTA (0.5M), and incubation continued for 18 minutes at 37°. Cells were harvested by centrifugation at 1600 x g for 30 minutes, resuspended in 3 mL SMMD and transferred to 50 mL of G medium. The sample was incubated at 37° for 4 hours, during which the Klett value increased. Klett values were measured with a Klett™ colorimeter (Manostat Manufacturing) set on the green filter. Spheroplasts were harvested by

centrifugation at 1600 x g for 30 minutes and resuspended in 3 mL SMMD. An aliquot was taken to determine efficiency of spheroplast formation as described below. Spheroplasts of each strain were mixed (2 mL total volume), concentrated by centrifugation at 1600 x g for 20 min. and resuspended in 0.2 mL SMMD before addition of 1.8 mL of PEG fusion solution. The control mixture was not treated with the PEG fusion solution. After 10 minutes at room temperature, 100 μL aliquots were plated on S-LBH agar plates supplemented with antibiotics appropriate for the selection and enumeration of parent cells and fusion transconjugants.

2.3.2.3. Spheroplast fusion following growth in the presence of ampicillin. To generate spheroplasts, cells from saturated cultures were diluted 1:100 with S-LBH supplemented with ampicillin and incubated at 37° with aeration for 3-4 hours. At this time cell density had visibly increased, confirmed by an increase in the Klett value. Spheroplasts were harvested by centrifugation at 1600 x g for 20 minutes, resuspended in 2 mL SMMD, and the efficiency of spheroplast formation was determined as outlined below. The resultant suspension was used in fusion experiments as detailed in section 2.2.2.2.

2.3.3. Determination of efficiency of spheroplast formation.

Spheroplast formation was monitored using light microscopy. A 10 μL sample of treated cells were observed under moderate magnification. Distilled water was added at the edge of the coverslip, and its diffusion was accompanied by rupture of cells in the spheroplast state. To quantitatively determine the efficiency of spheroplast formation after growth in G medium, the cultures were serially diluted separately in both S-LBH and distilled water and the dilutions plated on S-LBH agar plates. After overnight incubation, titres were calculated and the proportion of cells converted to spheroplasts determined.

3. RESULTS.

3.1. Efficiency of spheroplast formation.

3.1.1. Following lysozyme treatment.

The proportion of each culture converted into spheroplasts using the method of Reina et al (1993) was determined. The results are shown in Table 3.1. Spheroplast numbers were calculated by comparing titres in S-LBH dilution series with those in the distilled water dilution series. The difference in titre corresponded to the rupture of spheroplasts under osmotic pressure, and thus the proportion present in each culture.

Table 3.1. Proportion of spheroplasts formed after lysozyme-glycine treatment

strain	titre(S-LBH)	titre(dH_2O)	spheroplasts(%)
GR936	$2x10^7 \pm 3x10^6$	$5x10^5 \pm 8x10^4$	90-97
GR970	$4x10^7 \pm 6x10^6$	$8x10^5 \pm 2x10^5$	91-98

Titres represent an average value from 5 independent treatments

The extent of spheroplast formation was consistent with that reported by others (Reina et al, 1993, Marvin and Witholt, 1987).

3.1.2. Following incubation with ampicillin.

As seen in Table 3.2, ampicillin treatment was sufficient to produce spheroplasts to the same extent as observed for the lysozyme-glycine method.

Table 3.2. Proportion of spheroplasts formed after incubation with ampicillin

strain	titre(S-LBH)	titre(dH_2O)	spheroplasts(%)
GR970	$1x10^5 \pm 5x10^4$	$2x10^3 \pm 9x10^2$	94-98

Titres represent an average of 4 independent treatments

These figures are consistent with previously reported data for ampicillin-induced spheroplast formation (Marvin and Witholt, 1987).

3.2. Fusion between *E. coli* spheroplasts.

Two fusion protocols involving lysozyme treatment were carried out in an attempt to generate transformants through fusion-dependent transfer of non-self-transferable plasmids. Transformants were not recovered at a detectable frequency. Values of the maximum theoretical frequencies are presented in Table 3.3.

Table 3.3. Maximum theoretical transformation frequencies.

	protocol for spheroplast production	parent cell titre following growth to saturation		transformation frequency
		GR936	GR970	
1	lysozyme	$5x10^7 \pm 9x10^6$	$4x10^7 \pm 1x10^7$	$\leq 3x10^{-7}$
2	lysozyme-glycine	$2x10^7 \pm 6x10^6$	$3x10^7 \pm 6x10^6$	$\leq 5x10^{-7}$
3	ampicillin	$1x10^8 \pm 6x10^7$	$8x10^7 \pm 3x10^7$	$\leq 1x10^{-7}$

Transformation frequency is the theoretical maximum based on the titre of the limiting parent

The maximum frequency of the fusion event could have been as high as $5x10^{-7}$ per parent cell (row 2), similar to that previously reported (Reina et al, 1993). Transformants were not recovered despite a high rate of conversion of cells to spheroplasts prior to exposure to the fusogenic agent (Tables 3.1 and 3.2). Fusion after ampicillin treatment was also beyond detection (Table 3.3, row 3), even though the extent of spheroplast formation by this treatment was equivalent to that obtained with lysozyme treatment.

4. DISCUSSION.

4.1. Fusion between *E. coli* spheroplasts.

The results here show that both lysozyme treatment and exposure to ampicillin were responsible for conversion of cells to spheroplasts to an extent consistent with that reported previously (Marvin and Witholt, 1987, Reina et al, 1993). The failure to recover transformants using these fusion protocols may have had several causes.

The theoretical maxima calculated in Table 3.3 suggest that the frequency of transformation occurring via fusion was at best very low. However, these values may be misleading as they were generated from titres calculated following growth to saturation of fusion partners after the fusion event. It is possible that the cell titres during the fusion event were much less (perhaps more than an order of magnitude); this is consistent with titres calculated from spheroplast cultures immediately before fusion (Tables 3.1 and 3.2). If this is indeed the case, then it is possible that failure to recover transformants can be attributed to an insufficient number of spheroplast fusion partners during the fusion treatment.

A previous report of genetic exchange involving fusion of *E. coli* spheroplasts involved overnight growth in the presence of a subinhibitory concentration of polymyxin B (Reina et al, 1993). Reina et al, suggested that this treatment weakened the cell wall sufficiently to allow greater areas of inner membrane to be exposed by lysozyme treatment, with a subsequent increase in fusion. Their study was based on recovery of recombinants which had regained a chromosomal marker. It was reasoned here that plasmid transfer may not require as great an area for fusion, as the plasmids used were relatively small, so the polymyxin B treatment was not included. Furthermore, it was not known

what effect the combination of two cell wall active agents would have had on the cells. A further factor which may have contributed to the observed results was the stains used. As they were different to those used previously, the lysozyme treatment employed may have had a more severe effect. It is known that conversion to the spheroplast form can cause irreversible changes in cells which prevent the regeneration of the cell wall (Marvin and Witholt, 1987).

4.2. Future directions.

The failure to detect transformants using these fusion protocols may have been the result of insufficient cell numbers during the fusion event. This could possibly be overcome by increasing the number of cells exposed to the fusogenic agent. If fusion was still unsuccessful the requirement for polymyxin B for the fusion phenomenon could be determined. It would then be of interest to combine a preincubation with polymyxin B and a subsequent treatment with ampicillin in the fusion protocol. An alternative direction for future work in this area could involve an investigation into the transforming ability of the DNA-containing membrane vesicles produced by pathogenic gram-negative bacteria. As described earlier, these vesicles are thought to contain plasmid DNA and their production is increased in the presence of gentamicin (Kadurugamuwa et al, 1996). In addition *E. coli* spheroplasts can fuse with unilamellar lipid vesicles (Marvin et al, 1989). The combination of *E. coli* spheroplasts and MVs could conceivably lead to *E. coli* transformation and could be investigated using available protocols for collection of vesicles and fusion. As gentamicin and other aminoglycosides are often administered in conjunction with ampicillin or other β-lactams during antimicrobial therapy, the combined effects of these agents may be environmentally relevant to genetic exchange.

CHAPTER IV

CONCLUSION

The results presented here may have ramifications for the spread of antimicrobial resistance determinants. The influence of elevated mating temperature on the transmission of plasmids has the potential to affect their exchange in situ. The exposure of the normal human intestinal microbiota to antimicrobials results in establishment of a pool of resistant non pathogenic species, especially in long term hospital patients. This pool has been implicated in the appearance of resistant pathogens, due to exchange of broad host range plasmids between the commensal bacteria and opportunistic pathogens (Ewald, 1988). The danger implicit in this prediction is heightened by the occasional inattention of some health care workers to basic procedures designed to limit the spread of nosocomial infections (Doebbeling et al, 1992, Livornese et al, 1992). The increase in transmission frequency at elevated temperatures may provide a mechanism for intergeneric gene exchange. Increases in the body temperature of individuals experiencing fever may promote gene transmission.

In this study 43° was found to be optimal for interspecific transmission, although some increase was observed at normal fever temperatures. However, the environment within the human body could be more stressful than the experimental conditions employed. As environmental stresses other than temperature fluctuations can also lead to changes in restriction, combinations of such factors may collude with moderate temperature increases to potentiate plasmid transmission. It is also known that antimicrobial therapy involving gentamicin can lead to the production of membrane vesicles containing DNA

and virulence factors, and that other agents influence outer membrane permeability (Hancock et al, 1981, Kadurugamuwa and Beveridge, 1996). The DNA contained in released vesicles is in the closed circular form, probably plasmid, although the transforming ability of these vesicles has yet to be demonstrated. In conjunction with the elevated temperature of a patient suffering from a bacterial (or other) infection, antimicrobial therapy itself may help promote gene exchange.

My results suggest that there exists a barrier to the establishment of conjugatively transferred DNA other than that imposed by restriction nucleases. If this barrier is indeed related to the integrity of the cell envelope, perturbations in the outer membrane and cell wall may also potentiate gene exchange. Unfortunately the fixed time limit imposed on this work prevented the full exploration of the fusion phenomenon. It was possible however to show that exposure of recipient cells to Ca^{2+}, a treatment known to affect the cell envelope, could promote plasmid transmission by conjugation.

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Appendix I. Media

I.i. General Media

LBH
Tryptone 1% w/v
Yeast Extract 0.5% w/v
NaCl 0.5% w/v
1mM NaOH

LBH Agar
as above with 1.5% w/v agar

Nutrient Broth
beef extract 0.3% w/v
peptone 0.5% w/v

I.ii. Media for spheroplast generation/stabilisation

S-LBH
in 200mL:
100 mL 20% sucrose
90 mL LBH
10mL Tris (pH 8)

SOS
in 20 mL:
10 mL 2M sorbitol
6.7 mL LBH
3.17 mL dH_2O
0.13 mL $CaCl_2$

G medium
Nutrient Broth supplemented with:
sucrose (0.5M)
20% glycine
10mM $MgCl_2$
0.2% (w/v) $MgSO_4$
(Coetzee et al, 1979)

Appendix II. Buffers and Solutions

II.i. General Buffers

TNB
0.8% Tris-HCl
0.05% NaCl
pH 7.6

II.ii. DNA isolation buffers

Solution I
50mM glucose
25mM Tris
10mM EDTA

Solution II
1% w/v SDS
0.2N NaOH

Solution III
3M sodium acetate pH 4.8

II.iii. Buffers used in preparation of spheroplasts

SMMD
0.5M sucrose
0.2M maleate pH 6.5
20mM $CaCl_2$
added after autoclaving: DNaseI 20μgmL^{-1}
(Wyrick and Rogers, 1973)

II.iv Fusion solutions

Sherman et al
20% PEG 4000
10mM $CaCl_2$
10mM Tris-HCl
pH 7.4

Reina et al
40% w/v PEG 6000
0.5M sucrose
100mM $CaCl_2$
15% v/v dimethyl sulfoxide (filter sterilised)

Appendix III. Antibiotics

The antibiotics and working concentrations used in this study:

Antibiotic	**concentration (μg/mL)**
Ampicillin	100
Chloramphenicol	50
Kanamycin	30
Nalidixic acid	60
Rifampicin	100
Streptomycin	100
Tetracycline	20